L5: Entry 46 of 81 File: USPT Jun 20, 2000

DOCUMENT-IDENTIFIER: US 6077699 A
TITLE: Polypeptides involved in the biosynthesis of streptogramins, nucleotide sequences coding for these polypeptides and their use

Detailed Description Text (203):
250 g of a centrifugation pellet, washed with 0.1 M phosphate buffer pH 7.2, 1 mM PMSF, 5 mM EDTA, 5 mM EGTA, 0.5 M KCl, 10% v/v glycerol, of an S. pristinaespiralis SP92 culture harvested at the beginning of the pristinamycin production phase are taken up with 750 ml of pH 8.0 100 mM Tris-HCl buffer containing 4 mM DTE, 5 mM benzamidine, 0.2 mM Pefabloc, 1 mM EDTA, 1 mM EGTA, 2 mg/l leupeptin, 2 mg/l STI, 2 mg/l aprotinin, 1 mg/l E-64, 20% v/v glycerol. The suspension thereby obtained is ground using a French Press adjusted to a pressure of 5000 psi, and then centrifuged at 50,000 g for 1 h. The crude extract thereby collected is fractionated by ammonium sulphate precipitation. The protein fraction coming out at between 0 and 35% ammonium sulphate saturation is redissolved in the disruption buffer and desalted on a column of Sephadex G 25 Fine equilibrated and eluted in this same buffer. The proteins thus prepared are injected in pH 8.0 100 mM Tris-HCl buffer, 4 mM DTE, 2 mM benzamidine, 2 mg/l leupeptin, 1 mg/l E-64, 20% v/v glycerol onto a column (200 ml) of Q Sepharose Fast Flow, and are then eluted with a linear KCl gradient (0 to 0.6 M). At outflow from the column, each fraction is treated with one-tenth of its volume of a solution of 2 mM Pefabloc, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine. The fractions containing the enzymatic activity (detected by means of the test described in Example 5.4.1.A) are pooled and precipitated with ammonium sulphate at 80% saturation. The proteins which have come out are redissolved in pH 6.8 50 mM bis-tris propane buffer, 1 mM benzamidine, 1 mM DTE, 0.2 mM Pefabloc, 1 mM EDTA, 1 mM EGTA, 2 mg/l leupeptin, 0.15 M NaCl, 20% v/v glycerol, and injected in 5 4-ml aliquot portions onto a Superdex 200 Hi-Load 16/60 permeation column equilibrated and eluted in this same buffer. After assay, the active fractions are pooled and reconcentrated to 3 ml on Centriprep 30, then rediluted to 20 ml with pH 8.0 100 mM Tris-HCl buffer, 4 mM DTE, 1 mM benzamidine, 1 mM PMSF, 20% v/v glycerol and applied in two portions to a MonoQ HR 10/10 column equilibrated and eluted with a linear gradient from 0.4 M KCl in this same buffer. The best fractions containing the desired activity are pooled and used as material for characterization of the activities of the enzyme and for its microsequencing.

Detailed Description Text (223):
70 g of wet cells, harvested as described in Example 5.2.1.B., are resuspended in 250 ml of cell lysis buffer (100 mM Tris-HCl pH 8.0 containing 25% of glycerol, 4 mM DTE, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mg/l E-64, 2 mg/l STI, 2 mg/l .alpha..sub.2 -macroglobulin, 1 mg/l leupeptin, 2 mg/l aprotinin, 5 mM benzamidine, 0.6 mg/ml lysozyme. The solution thereby obtained is kept stirring at 4.degree. C. for 1 h and then centrifuged at 50,000 g for 1 h. The supernatant is then injected in the cell lysis buffer onto a column of Sephadex G-25, and the excluded fraction (approximately 250 mg of protein injected in each chromatographic run) is injected onto a column of Mono Q HR 16/10 (Pharmacia) equilibrated with 100 mM Tris-HCl buffer pH 8.0, 4 mM DTE, 1 mM EGTA, 1 mM EDTA, 1 mg/l E-64, 2 mg/l STI, 20% glycerol. The proteins are eluted with a linear gradient of from 0 to 0.6 M KCl and, at outflow from the column, each fraction is treated with one-tenth of its volume of a solution of 2 mM Pefabloc, 5 mM EGTA, 5 mM EDTA. The fractions containing the activity are pooled and then mixed with 1 volume of 100 mM Tris-HCl pH 8.0 15% glycerol, 1 mM PMSF, 1 mM benzamidine, 4 mM DTT, 3.4 M ammonium sulphate per 3 volumes of fraction. The solution is injected onto a column of Phenyl Superose HR

10/10 (one-fifth of the solution is injected at each chromatographic run), and the proteins are eluted with a decreasing linear gradient of from 0.9 to 0 M ammonium sulphate. The fractions containing the activity are pooled. The solution is concentrated to 3500 .mu.l in a Centriprep 30 and injected in two portions onto a Superdex 200 Hi-Load 16/60 column equilibrated and eluted with 50 mM bis-tris propane buffer pH 6.8 containing 20% of glycerol, 0.15 M NaCl, 4 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 mM EDTA. The active fraction is diluted with 9 volumes of 50 mM bis-tris propane buffer pH 6.8 containing 25% of glycerol, 4 mM DTT, 1 mM PMSF, 1 mM benzamidine, and then injected onto a column of Mono Q HR 5/5 equilibrated in the same buffer. The desired activity is eluted with a linear gradient of from 0 to 0.4 M KCl and concentrated to 630 .mu.l in a Centricon-30. The desired protein is then purified by electrophoresis on 6% polyacrylamide gel after denaturation of the sample by heating for 10 min at 80.degree. C. with an SDS/mercaptoethanol mixture. After electrophoresis and staining of the gel with Coomassie blue, the gel band containing the protein is cut out and the protein is electroeluted from the gel in a Centrilutor.

L5: Entry 50 of 81 File: USPT Apr 6, 1999

DOCUMENT-IDENTIFIER: US 5891695 A
TITLE: Polypeptides involved in the biosynthesis of streptogramins, nucleotide sequences coding for these polypeptides and their use

Detailed Description Text (207):
250 g of a centrifugation pellet, washed with 0.1M phosphate buffer pH 7.2, 1 mM PMSF, 5 mM EDTA, 5 mM EGTA, 0.5M KCl, 10% v/v glycerol, of an S. pristinaespiralis SP92 culture harvested at the beginning of the pristinamycin production phase are taken up with 750 ml of pH 8.0 100 mM Tris-HCl buffer containing 4 mM DTE, 5 mM benzamidine, 0.2 mM Pefabloc, 1 mM EDTA, 1 mM EGTA, 2 mg/l leupeptin, 2 mg/l STI, 2 mg/l aprotinin, 1 mg/l E-64, 20% v/v glycerol. The suspension thereby obtained is ground using a French Press adjusted to a pressure of 5000 psi, and then centrifuged at 50,000 g for 1 h. The crude extract thereby collected is fractionated by ammonium sulphate precipitation. The protein fraction coming out at between 0 and 35% ammonium sulphate saturation is redissolved in the disruption buffer and desalted on a column of Sephadex G 25 Fine equilibrated and eluted in this same buffer. The proteins thus prepared are injected in pH 8.0 100 mM Tris-HCl buffer, 4 mM DTE, 2 mM benzamidine, 2 mg/l leupeptin, 1 mg/l E-64, 20% v/v glycerol onto a column (200 ml) of Q Sepharose Fast Flow, and are then eluted with a linear KCl gradient (0 to 0.6M). At outflow from the column, each fraction is treated with one-tenth of its volume of a solution of 2 mM Pefabloc, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine. The fractions containing the enzymatic activity (detected by means of the test described in Example 5.4.1.A) are pooled and precipitated with ammonium sulphate at 80% saturation. The proteins which have come out are redissolved in pH 6.8 50 mM bis-tris propane buffer, 1 mM benzamidine, 1 mM DTE, 0.2 mM Pefabloc, 1 mM EDTA, 1 mM EGTA, 2 mg/l leupeptin, 0.15M NaCl, 20% v/v glycerol, and injected in 5 4-ml aliquot portions onto a Superdex 200 Hi-Load 16/60 permeation column equilibrated and eluted in this same buffer. After assay, the active fractions are pooled and reconcentrated to 3 ml on Centriprep 30, then rediluted to 20 ml with pH 8.0 100 mM Tris-HCl buffer, 4 mM DTE, 1 mM benzamidine, 1 mM PMSF, 20% v/v glycerol and applied in two portions to a MonoQ HR 10/10 column equilibrated and eluted with a linear gradient from 0.4M KCl in this same buffer. The best fractions containing the desired activity are pooled and used as material for characterization of the activities of the enzyme and for its microsequencing.

Detailed Description Text (229):
70 g of wet cells, harvested as described in Example 5.2.1.B., are resuspended in 250 ml of cell lysis buffer (100 mM Tris-HCl pH 8.0 containing 25% of glycerol, 4 mM DTE, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 1 mg/l E-64, 2 mg/l STI, 2 mg/l .alpha..sub.2 -macroglobulin, 1 mg/l leupeptin, 2 mg/l aprotinin, 5 mM benzamidine, 0.6 mg/ml lysozyme. The solution thereby obtained is kept stirring at 4.degree. C. for 1 h and then centrifuged at 50,000 g for 1 h. The supernatant is then injected in the cell lysis buffer onto a column of Sephadex G-25, and the excluded fraction (approximately 250 mg of protein injected in each chromatographic run) is injected onto a column of Mono Q HR 16/10 (Pharmacia) equilibrated with 100 mM Tris-HCl buffer pH 8.0, 4 mM DTE, 1 mM EGTA, 1 mM EDTA, 1 mg/l E-64, 2 mg/l STI, 20% glycerol. The proteins are eluted with a linear gradient of from 0 to 0.6M KCl and, at outflow from the column, each fraction is treated with one-tenth of its volume of a solution of 2 mM Pefabloc, 5 mM EGTA, 5 mM EDTA. The fractions containing the activity are pooled and then mixed with 1 volume of 100 mM Tris-HCl pH 8.0, 15% glycerol, 1 mM PMSF, 1 mM benzamidine, 4 mM DTT, 3.4M ammonium sulphate per 3 volumes of fraction. The solution is injected onto a column of Phenyl Superose HR

10/10 (one-fifth of the solution is injected at each chromatographic run), and the proteins are eluted with a decreasing linear gradient of from 0.9 to 0M ammonium sulphate. The fractions containing the activity are pooled. The solution is concentrated to 3500 .mu.l in a Centriprep 30 and injected in two portions onto a Superdex 200 Hi-Load 16/60 column equilibrated and eluted with 50 mM bis-tris propane buffer pH 6.8 containing 20% of glycerol, 0.15M NaCl, 4 mM DTT, 1 mM PMSF, 1 mM benzamidine, 1 mM EDTA. The active fraction is diluted with 9 volumes of 50 mM bis-tris propane buffer pH 6.8 containing 25% of glycerol, 4 mM DTT, 1 mM PMSF, 1 mM benzamidine, and then injected onto a column of Mono Q HR 5/5 equilibrated in the same buffer. The desired activity is eluted with a linear gradient of from 0 to 0.4M KCl and concentrated to 630 .mu.l in a Centricon-30. The desired protein is then purified by electrophoresis on 6% polyacrylamide gel after denaturation of the sample by heating for 10 min at 80.degree. C. with an SDS/mercaptoethanol mixture. After electrophoresis and staining of the gel with Coomassie blue, the gel band containing the protein is cut out and the protein is electroeluted from the gel in a Centrilutor.

L5: Entry 67 of 81 File: USPT Oct 15, 1991

DOCUMENT-IDENTIFIER: US 5057417 A
TITLE: Compositions and methods for the synthesis of growth hormone receptor and growth hormone binding protein

Detailed Description Text (177):
Therefore, a multi-step screening process would be used. Antibodies to the binding protein would be obtained. Animals are immunized against the binding protein or to selected fragments thereof in conjugate form with such proteins as keyhole limpet hemoganin (KLH), bovine serum albumin (BSA), soybean trypsin inhibitor (STI) or bovine thyroglobulin (BT) by combining 1 mg or 1 .mu.g of binding protein or conjugate (for rabbit or mice, respectively) with 3 volumes of Freund's complete adjubant and injecting the solution intradermally at multiple sites. Booster shots of 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites are given one month after the initial immunization. Animals are bled one to two weeks following the booster and the serum assayed for anti-growth hormone receptor. Animals are boosted until the titer plateaus. Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by EB virus transformation and screening for clones expressing the desired antibody. Such an antibody could be incorporated into a standard immunoassay (ELISA, RIA, etc) to determine the level of binding protein in the serum. In its simplest form, this assay would indicate if normal amounts of liver receptors are present, but it could be further refined by judicious selection of monoclonal antibodies to distinguish normal from defective types of binding protein. If the binding protein proved normal, then a further screen comparing the correct gene sequence to the patient's DNA using standard restriction mapping techniques could be used to identify defects in other portions of the receptor.

L5: Entry 55 of 81 File: USPT Nov 18, 1997

DOCUMENT-IDENTIFIER: US 5688763 A
TITLE: Compositions and methods for the synthesis of growth hormone receptor and growth hormone binding protein

Detailed Description Text (177):
Therefore, a multi-step screening process would be used. Antibodies to the binding protein would be obtained. Animals are immunized against the binding protein or to selected fragments thereof in conjugate form with such proteins as keyhole limpet hemoganin (KLH), bovine serum albumin (BSA), soybean trypsin inhibitor (STI) or bovine thyroglobulin (BT) by combining 1 mg or 1 .mu.g of binding protein or conjugate (for rabbit or mice, respectively) with 3 volumes of Freund's complete adjubant and injecting the solution intradermally at multiple sites. Booster shots of 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites are given one month after the initial immunization. Animals are bled one to two weeks following the booster and the serum assayed for anti-growth hormone receptor. Animals are boosted until the titer plateaus. Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by EB virus transformation and screening for clones expressing the desired antibody. Such an antibody could be incorporated into a standard immunoassay (ELISA, RIA, etc.) to determine the level of binding protein in the serum. In its simplest form, this assay would indicate if normal amounts of liver receptors are present, but it could be further refined by judicious selection of monoclonal antibodies to distinguish normal from defective types of binding protein. If the binding protein proved normal, then a further screen comparing the correct gene sequence to the patient's DNA using standard restriction mapping techniques could be used to identify defects in other portions of the receptor.

L5: Entry 56 of 81 File: USPT Oct 28, 1997

DOCUMENT-IDENTIFIER: US 5681571 A
TITLE: Immunological tolerance-inducing agent

Detailed Description Text (34):
group 2:0.5 ml of 0.6M bicarbonate buffer containing 5 mg MBP and 10 mg of soybean trypsin inhibitor (STI) to minimize proteolytic degradation of MBP (Whitacre, C. C., Gienap, I. E., Orosz, C. G., Bitar, D. 1991. Oral tolerance in experimental autoimmune encephalitis. III. Evidence for clonal anergy. J. Immunol. 147:2155-63), 5 consecutive times on day -11, -9, -7, -5, and -3 before footpad injection with MBP;

Detailed Description Text (38):
Groups consisting of 5 adult female Lewis rats were injected in the rear footpads with MBP emulsified in FCA or with FCA alone. In control animals previously fed with soybean trypsin inhibitor (STI) (group 4), given 11, 9, 7, 5 and 3 days before footpad injection with MBP+FCA, neurological symptoms developed being maximal 12 to 14 days after the injection and all animals developed severe paralysis (Table 8). Animals fed as little as 20 micrograms of MBP conjugated to CTB administered in a single dose (group 1), developed either no (4 out of 5 rats) or mild symptoms (transient tail paresis associated with right hind footpad paresis in another rat) (Table 8). Animals fed 5 milligram of MBP together with STI on 5 consecutive occasions, that is a total of 25 mg MBP or 1250 times higher doses of MBP than group 1 animals, were also protected from developing severe EAE disease (group 2) (Table 8). Thus, oral administration of small amounts of MBP conjugated to CTB can suppress EAE.

Detailed Description Paragraph Table (7):
TABLE 8 _____ Suppression of experimental autoimmune encephalitis (EAE) in Lewis rats by oral administration of myelin basic protein conjugated to CTB clinical incidence animal number of footpad score of of group feeding doses injection EAE paralysis.sup.a
_____ 1 MBP (20 1 MBP + FCA 1.1 .+-. 0.4 0/5 .mu.g)-CTB 2 MBP 5 mg 5 MBP + FCA 0.6 .+-. 0.5 0/5 (+STI) 3 saline 5 FCA 0 0/5 4 STI 5 MBP + FCA 3.9 .+-. 0.5 5/5 _____ .sup.a Clinical grade .gtoreq. 2

L5: Entry 5 of 5 File: USPT Nov 27, 2001

DOCUMENT-IDENTIFIER: US 6323219 B1
TITLE: Methods for treating immunomediated inflammatory disorders

Detailed Description Text (17):
More particularly, in our opinion, some of the compounds of the foregoing formula containing a D-phenylalanine-proline-arginine motif should be effective in inhibiting the PAR-2 pathway and causing depigmentation. One particularly preferred compound which acts as a thrombin, trypsin and tryptase inhibitor and is active in depigmenting mammalian skin is (S)N-Methyl-D-phenylalanyl-N-[4-[(aminoiminomethyl)amino]-1-(2-benzothiazolylcarbonyl)butyl]-L-prolinamide (Chemical Abstracts name) (hereinafter referred to as "Compound I"). Other compounds which are analogs or function similarly to Compound I and are set forth in U.S. Pat. No. 5,523,308 are active in the methods and compositions of this invention. Other compounds that inhibit trypsin, such as serine protease inhibitors, and in particular, soybean trypsin inhibitor (STI) will also be useful in methods of this invention. Soybean, limabean and blackbean extracts, and other natural products made from these beans, such as, but not limited to, bean milk, bean paste, miso and the like, also serve to reduce pigmentation by the PAR-2 pathway.

Detailed Description Text (28):
We have unexpectedly found that when topically active agents, such as PAR-2 agonists and/or inhibitors and trypsin and/or thrombin and/or tryptase and/or their inhibitors, are topically applied to an animal's skin, a significant change in pigmentation was achieved. Preferably, depigmenting agents (as well as other pigmentation-affecting agents of this invention) are applied to the skin of a mammal at a relatively high concentration and dose (from about 0.005% to about 1% for compounds having high therapeutic indices such as Compound I and related compounds; from about 20% to about 99% for liquid derivatives and extracts of botanical materials; and from about I% to about 20% for fractions of natural extracts and naturally-derived protease inhibitors such as STI or mixtures thereof) between one and two times daily for a period of time until the skin evidences a change in pigmentation. This may be for from about four to about ten weeks or more. Thereafter, once the change in pigmentation has been achieved, a lower concentration and dose (from about 0.00001% to about 0.005% for compounds having high therapeutic indices such as Compound I and related compounds; from about 10% to about 90% for liquid derivatives and extracts of botanical materials; and from about 0.01% to about 5% for fractions of natural extracts and naturally-derived protease inhibitors such as STI or mixtures thereof), of active ingredient may be applied on a less frequent time schedule, e.g., about once per day to about twice per week. The effects of the active agents of this invention are reversible, therefore, in order to maintain these effects, continuous application or administration should be performed. The invention illustratively disclosed herein suitably may be practiced in the absence of any component, ingredient, or step which is not specifically disclosed herein.

Detailed Description Text (60):
TRP-1 and TRP-2 are melanocyte-specific. Compound I inhibits trypsin and thrombin. Hirudin, a specific thrombin inhibitor, had no effect on pigmentation, as seen above in Example 2. Thus, we decided to test whether trypsin and thrombin are expressed in skin. A probe designed to detect both brain and gastric trypsins, as described in

Table C, detected the expression of both mRNAs in a total skin mRNA sample available from Invitrogen of Carlsbad, Calif., as well as in MelanoDerm equivalents. The same expression pattern was detected for thrombin. Both trypsin and thrombin were not expressed in normal melanocytes (FIGS. 5A, B). These data suggest that if trypsin activates PAR-2, it could be produced by the keratinocytes only. As shown in FIG. 6A, treatment with Compound I resulted in increased expression of trypsin. SLIGRL, which did not affect melanogenesis gene expression (FIG. 6B) also increased trypsin expression in the equivalents. We conclude that while trypsin is a possible natural activator of PAR-2 in skin and possibly affects pigmentation, its mRNA levels do not correlate with pigmentation. This suggests that another, yet unidentified serine protease, which is inhibited by compound 1, STI and the like, is the natural activator of PAR-2 in the epidermis. Compounds that induce or inhibit this protease would serve as darkening and lightening agents, respectively.

Detailed Description Text (82):
Soybean milk, soybean paste and miso were prepared to be used as naturally-derived materials that contain STI and are able to lighten skin color.

Detailed Description Text (85):
Two Yucatan swine were treated for eight and ten weeks, twice a day, five days a week, with different soybean- and lima-bean-derived products. These natural products include soybean paste, soybean protein acid hydrolysate, miso, native and boiled soybean milk, and a commercially available extract of soybean (Actiphyte.TM. of Active Organics, Dallas Tex.), as well as purified STI, and different preparations of trypsin inhibitors from soybeans and limabeans. At seven weeks of treatment, all sites were visually lighter than the surrounding skin, except for the boiled soybean milk and the soybean protein acid hydrolysate treated sites. Histological analysis of biopsies from the treated sites following F&M staining confirmed the depigmenting effect of the soybean and limabean products. An example of such histological data is given in FIG. 13. The lack of depigmenting activity in the boiled soybean milk and in the soy protein acid hydrolysate is explained by the denaturation or the degradation of the soy proteins in these preparations, respectively. We theorize that the active depigmenting agents in the soybean and limabean products are soybean trypsin inhibitor (STI) and limabean trypsin inhibitor, respectively. (Example 1 shows the depigmenting effect of STI in vitro). This example demonstrate that natural extracts containing trypsin inhibitory activity could be used as whitening agents which affect the PAR-2 pathway.

Detailed Description Text (90):
As shown in FIG. 14, the age spot treated with STI became lighter following 8 weeks of treatment. FIG. 14 is a composite of four pictures. The left panel is the visible light pictures of the hand, before (upper) and after (lower) 8 weeks of treatment. At this orientation the top age spot is the placebo-treated, the middle age spot is untreated, and the lower age spot is the STI-treated. The right panel shows the same hand at the same time points, using UV-photography. UV light enables the visualization of pigment deeper in the skin, demonstrating that the STI whitening effect was not superficial. FIG. 14 clearly demonstrates that the STI formulation was able to lighten the lower age-spot. An increase of 15 L* units was calculated for this STI-treated site, further demonstrating the ability of this treatment to lighten age spots.

Detailed Description Text (95):
STI, soybean paste and other trypsin inhibitor-containing natural extracts can be incorporated into such formulations to provide increasing concentrations of the serine protease inhibitor. Use levels of the added active ingredient can range between 0.01% to 15% in a formulation. Other depigmenting agents, including PAR-2 inhibitors, tyrosinase inhibitors, hydroquinones, soy products, ascorbic acid and its derivatives, as well as other ingredients with skin care benefits could also be incorporated into this formulation.